

Antioxidative properties of a traditional tincture and several leaf extracts of *Allium ursinum* L. (collected in Montenegro and Bosnia and Herzegovina)

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Abstract

In order to estimate the amount of total phenols, flavonoids and the antioxidative properties of a traditional tincture, methanolic and ethanolic extracts from dried leaves of two different ecotypes of wild garlic (*Allium ursinum* L.), Folin-Ciocalteu method, AlCl₃ method and DPPH radical scavenging assay were used. It was found that the type of extraction solvent affects the value of the detected bioactive compounds. The highest TPC, total flavonoid content and DPPH antiradical activity were found in 80% methanolic extract, 2111,11 mg GAE/100g DW, 20,00 mg QE/1g DW and EC₅₀=77,21µg/ml, while the results obtained from the traditional tincture and 70% ethanolic extract were almost equal values. Significant correlation between the antioxidant activity and total flavonoid content ($p>0.05$; $r=0.99$) was found for a traditional tincture

1. Introduction

Allium is the largest and the most important representative genus of the *Alliaceae* family widely distributed in the northern hemisphere, North America, North Africa, Europe and Asia [1]. At the present time, the *Allium* family has over 600 members, each differing in taste, form and color, but close in biochemical, phytochemical and nutraceutical content [2].

Besides the well known garlic and onion, several other species are widely grown for culinary use, as a flavoring vegetable in various types of food due their flavor, aroma and taste [3], and for folk medicine, as an antihypertensive, antiatherosclerotic, antimicrobial, antidiarrhoeal and antiphlogistic agents [4]. Among them is wild or bear's garlic (*Allium ursinum* L.) [1] which botanical, phytochemical and pharmacological characteristics was overviewed by Sabolewska et al. [4]. *Allium ursinum* L. was called "the new star" of garlic in the German health journal *Therapiewoche* (Therapy week) and in 1992. was declared European medicinal "Plant of the year" by the Association for the Protection and Research on European Medicinal plants [5].

Several biological activities of *A. ursinum* L. plants, such as antioxidative, cytostatic, and antimicrobial, were reported [6]. Researchers have reported an increasing interest in *Allium* spp., because of its high antioxidative properties [7] which are well documented [8]. These properties are due to many substances, including some vita-mins, terpenoids, carotenoids, phytoestrogens, minerals, volatiles compounds and flavonoids [7].

In free radicals caused pathology these antioxidants neutralize free radicals [3], preventing the damage done to cells by free radicals-molecules that are released during the normal metabolic process of oxidation [9]. Studies around the world have identified many new plant constituents with antioxidant activity, among these are the polyphenols. The antioxidant activity of polyphenols has been reported to be mainly due to their redox properties, which can play an important role in neutralizing free radical and quenching oxygen, or decomposing peroxides [9].

A more recent study found that extract of the leaves of *A. ursinum* L. had strong antioxidant activity especially due to the high content of flavonoids [10]. Flavonoids and phenyl-acids have strong antioxidant properties, which makes them an important addition to the human diet [3]. They are a group of polyphenolic compounds isolated from a wide range of plants. Studies found that consumption of flavonoid-rich foods is associated with a lower incidence of heart disease, stroke, cancer and other chronic diseases. The literature

reports that flavonoid components reduce LDL oxidation and act as vasodilators of coronary arteries [11]. *Allium* species are amongst the richest sources of dietary flavonoids and contribute to a large extent to their overall intake [10].

The aims of this paper were to investigate bioactive compounds in the traditional tincture, methanolic and ethanolic extract of *Allium ursinum* L. leaves, and to estimate the amount of total phenols, total flavonoids and antioxidative potential, which is supposed to be high mainly due to the presence of flavonoid constituents

2. Experimental

2.1. Plant samples

Wild "srijemuš", *Allium ursinum* L. was collected in spring 2014. from two different locations – Gornje Lipovo (Kolašin, Montenegro) and Čemerno (Bosnia and Herzegovina). Fresh plant leaves were hand selected and used freshly (for the traditional tincture preparation) and dried (for methanolic and ethanolic extracts preparation).

2.2 Chemicals and reagents

Folin-Ciocalteu's and DPPH reagent were purchased from ALDRICH Chemistry. Solutions of 7,5% sodium carbonate, 2,5% $AlCl_3 \cdot 6H_2O$, and 10% sodium carbonate were freshly prepared. Gallic acid, quercetin and vitamin C were used as standards.

2.3 Instruments

UV/VIS spectrophotometer CECIL CE 2021 with quartz, 10mm width, and with transmission of 83% on 200 nm.

2.4 Extraction method

2.4.1. Methanolic and ethanolic extract

After collecting, fresh plant leaves were dried in a mild shadow and then grinded in a house blender. Grinded samples were stored in glass containers in a dark place at room temperature until use.

For ethanolic and methanolic extracts preparation we used modification of an extract preparation by classical maceration as described by Gitin et al. [7]. 6 g of dried leaf sample was extracted with 30 mL of 70% ethanol / 80% methanol by maceration in a ceramic mortar. When the material was homogenized, it was poured in plastic test tubes and centrifuged on a 4000 rpm for 10 minutes, on a temperature between 1 and 4°C.

2.4.2. Traditional tincture

This method of extraction was based on the traditional application of the studied plant. The recipe was obtained from Mr. Nikola Falak, a tincture producer from Bosnia and Herzegovina.

Freshly collected *Allium ursinum* L. leaves were cut into thin, small pieces and put in a transparent glass bottle. After putting the material, 1 L of 40% alcohol, with no sugar added, was poured, and the bottle was strongly closed by fastening its cork with the use of sticky tape. The bottle was held closed for 15 sunny days, and was periodically shaken. After 15 days the content was filtered in a dark-glass bottle and was kept in a cold, dark place until analyzing.

2.5 Determination of total phenolic and total flavonoid content

2.5.1. Folin-Ciocalteu's method

The total phenolics in the extract were determined using Folin-Ciocalteu method as described by Habila et al. [9]. All measurements were run in duplicate with gallic acid used as calibration standard. Results were expressed as gallic acid equivalents (mg GAE) per 100 g of dry weight. In a short description of the method 1.5 mL of Folin-Ciocalteu stock solution was mixed with 1.5 mL of 7.5% $NaHCO_3$ and then 200 μ L of investigated sample was added. Test tubes were kept in a room temperature for 30 minutes. After that, the absorbance was read on a wave light of 765nm. Test tube with no investigated sample was used as blank [12].

2.5.2. $AlCl_3$ method

The total flavonoid content in extracts and the traditional tincture was determined by a spectrophotometric method based on the formation of complex flavonoid-aluminium with an absorption maximum between 420-430 nm [13, 14]. Briefly, 1mL of 2.5% $AlCl_3 \cdot 6H_2O$ was added, and then it was mixed with 1 mL NaOH, 2 mL 10% Na-acetate and 6 mL of 70% ethanol. All the reagents were added with a time interspace of 5 minutes.

Finally, 1mL of prediluted sample was added. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixtures was measured at 420 nm. The flavonoid content values were determined from a standard curve prepared with quercetin (ranging from 10 to 50 µg/mL final volume) and expressed as mg quercetin equivalents (QE)/1g of dry weight.

2.6 Antioxidant activity

2.6.1. DPPH radical scavenging assay

Antioxidant activity was determined with the use of DPPH radical scavenging method according to Iliev et al. [8] which is a modification of the method described in Brand-Williams et al. [15]. In a test tube were added consequently – 1 mL of 0.9 µM DPPH solution, 3 mL of 70% ethanol and 100 µL of prediluted extract. In blank samples, extract was substituted by methanol. All test tubes were placed in dark box for 30 min at temperature 25°C, for conducting reaction, and absorption at 515nm was measured. Calibration of method was made with distilled water solution of ascorbic acid in concentration range between 0.1 and 0.9 g/L. Antiradical activity (AA) was calculated according to formula: $AA = ((A_{control} - A_{sample})/A_{control}) \times 100$, where the: $A_{control}$ is the absorbance of DPPH stock solution + methanol, A_{sample} is the absorbance of the DPPH stock solution + sample (or standard solution). The results were expressed as EC50 (concentration of extract or a referent substance, which is needed for inhibition of 50% of the DPPH radical). AA value is than compared with vitamin C as a referent substance.

2.7 Statistical analysis

The results were expressed as mean ± Standard Error. EC50 values were also calculated by linear regression analysis. Experiments results were further analyzed for Pearson correlation coefficient (r) between total phenolic, flavonoid and DPPH radical scavenging assay using the Statistica 7 software.

3. Results and discussion

3.1. Determination of total phenolic content

In our study total phenolic content of *Allium ursinum* L. dried leaf extracts for the ecotype Čemerno, was in the range of 1305,55 to 1833,33 mg GAE/100g DW (on average 1606,47mg GAE/100g DW). For the ecotype Gornje Lipovo, we have found the values in the range of 1027,77 to 2111,11 mg GAE/100g DW (on average 1402,77 mgGAE/100g DW) (Figure 1).

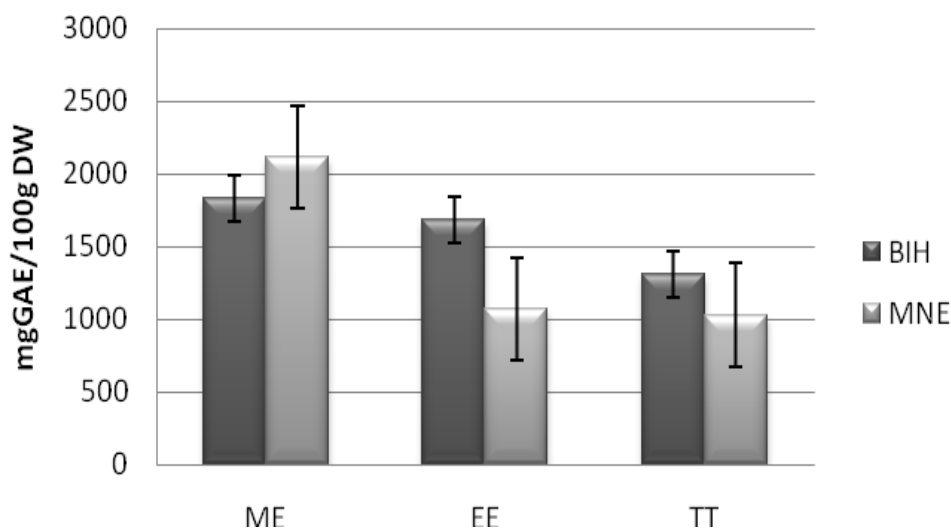


Figure 1: Total phenolic content of wild garlic dried leaf extracts (*Data expressed using Error bars with Standard Error of analyses of each type of extract; ME – methanol extract; EE - ethanol extract; TT - traditional tincture; DW - dry weight, BIH – (Čemerno, Bosnia and Herzegovina); MNE – Gornje Lipovo (Kolašin, Montenegro)).

in the range of 13,75 to 20,00 mgQE/1g DW. The results for *Allium ursinum* L., ecotype Gornje Lipovo, are in the range of 2,50 to 6,87 mgQE/1g DW (Figure 2).

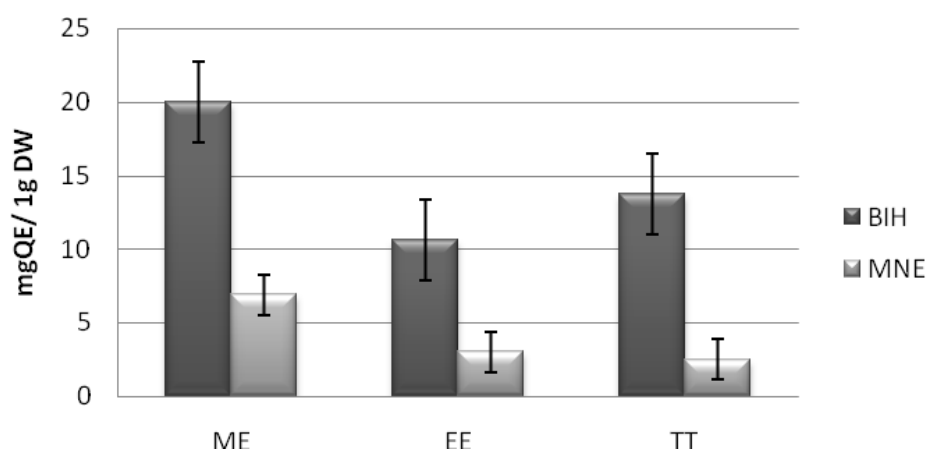


Figure 2: Total flavonoid content of wild garlic dried leaf extract (*Data expressed using Error bars with Standard Error of the analyses of each type of extract; ME – methanol extract; EE - ethanol extract; TT - traditional tincture; DW – dry weight; BIH – Čemerno (Bosnia and Herzegovina); MNE – Gornje Lipovo (Kolašin, Montenegro)).

3.3. Determination of antioxidant activity

The results showed that the antioxidant activity of *Allium ursinum* L. ecotype Čemerno was in the range of EC₅₀=50,00 µg/mL to EC₅₀=77,21 µg/mL, while the results for *Allium ursinum* L., ecotype Gornje Lipovo, were in the range of EC₅₀=44,99 µg/mL to EC₅₀=66,66 µg/mL (Figure 3.).

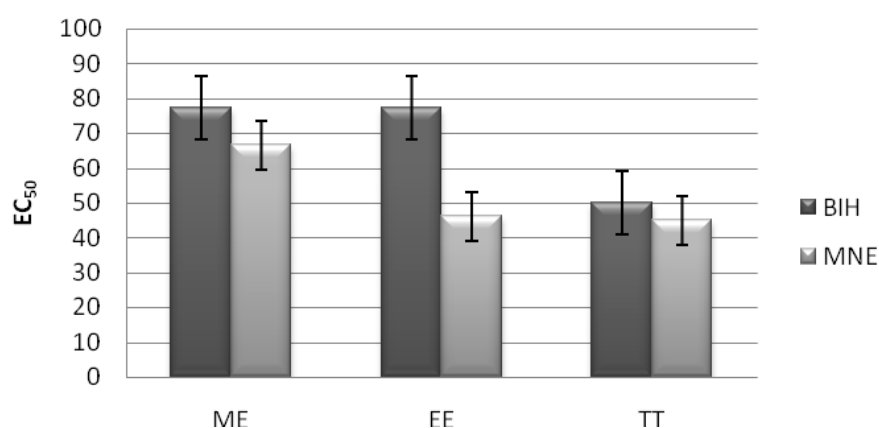


Figure 3: DPPH radical scavenging activity of *Allium ursinum* L. (*Data expressed using Error bars with Standard Error of the analyses of each type of extract; ME – methanol extract; EE - ethanol extract; TT - traditional tincture; DW - dry weight; BIH – Čemerno (Bosnia and Herzegovina); MNE – Gornje Lipovo (Kolašin, Montenegro)).

3.4. Total phenolic content

Djurđević et al. [16] have examined phenolic compounds in the leaves and inflorescences of *Allium ursinum* L. Alexieva et al. [17] obtained the total phenolics results ranged from 0.40 ± 0.02 to 0.41 ± 0.08 mg GAE/g FW and the values of polyphenolic content in the *A.ursinum* L. extract were established to be - 0.40 mg GAE/g FW Blazewicz-Wozniak and Michowska [18], who have studied three different ecotypes of wild garlic, found that the content of phenolic acids on average amounted to 713.7 mg×100 g-1 DW of leaves.

Keran et al. [19] studied total phenols content in three samples of dried wild onions from Bosnia and Herzegovina, and the calculated mean was 562.44 expressed as milligrams of gallic acid per 100 grams of fresh material. The highest value of the phenol content in the samples of raw material was 571, 12 mg GAE/g FW, while the lowest content was 552, 89 mg GAE/g FW.

Among our analysed samples, 80% methanolic extracts showed particularly high total phenolic values. The results of these determinations expressed as gallic acid equivalent (GAE) show a content of 1833,33 mg GAE /100g DW (Gornje Lipovo - Kolašin, Montenegro) and 2111,11 mg GAE /100g DW (Čemerno – Bosnia and Herzegovina) (Figure 1.) Our results ranged from 1027,77 to 2111,11 mg GAE/100g DW, which is lower than Keran et al. [19] results for fresh leaves extract. Our results concerning the *A. ursinum* L. are quite similar to the results from several studies conducted by Keran et al. [19] and Blazewicz-Wozniak and Michowska [18].

The highest phenolic values were found in the *A. ursinum* L. methanolic extract, while the lowest phenolic content showed the traditional tincture. Our study confirmed that this was the rule for both studied ecotypes. Ecotype Čemerno showed higher total phenolic values.

The quality and quantity of the biologically active compounds from *Allium* species significantly depend on the species, the plant organ and the harvest time. In *A. ursinum* L. leaves and bulbs, the highest amount of volatile precursors was found in March and April, shortly before flowering time [20]. The literature reports that the content of biologically active substances in the leaves of bear's garlic depended largely on its ecotype [18], what was also confirmed in our study.

Although phenolic compounds are not dominant secondary metabolites in the chemical composition of the garlic family [21] we have studied polyphenols due to the fact they are the most significant compounds for the antioxidant properties of plant raw materials. Then antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin [5].

3.5. Total flavonoid content

Blazewicz-Wozniak and Michowski [18], who have also investigated two different *Allium ursinum* L. ecotypes, reports that the sum of all flavonoids in *A. ursinum* L. leaves varied from 0.3185 g×100 g⁻¹ leaf D.W. of Dukla *A. ursinum* up to 0.3429 g in the Roztocze ecotype. They have found that on average, there was 0.3293 g of flavonoids expressed as quercetin equivalents in 100 grams of dry leaf mass. According to Djurdjević et al. [5], the leaves of bear's garlic contain 3.24 mg of flavonoids per gram.

Our study confirms that the sum of total flavonoids highly depends on plants ecotype and the extraction solvent. For *A. ursinum* L., flavonoid content was lower than the sum of total phenolics. 80% methanol was better extraction solvent than the 70% ethanol, which showed the highest flavonoid content (20,00 mg QE /1g DW for the ecotype Čemerno, and 6,87 mg QE /1g DW for the ecotype Gornje Lipovo). The lowest flavonoid content was detected in the traditional tincture – 2,50 mg QE/1g DW for the ecotype Čemerno, and 13,75 mg QE/1g for the ecotype Gornje Lipovo.

Flavonoids and phenyl-acids have strong antioxidant properties, which makes them an important addition to the human diet [3]. Due to the presence of those compounds in the studied extracts the antioxidant activity was also studied.

3.6. Antioxidative potential

Medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity [5]. The results shows that *Allium ursinum* L. extracts may have hydrogen donors thus scavenging the free radical DPPH. DPPH is a relatively stable Nitrogen centered free radical that easily accepts an electron or hydrogen, it react with suitable reducing agents as a results of which the electrons become paired off and the solution losses color depending on the number of electrons taken up [22]. The DPPH assay is commonly used for fast evaluation of the antioxidant capacity due to the simplicity of the assay [17].

The percentage of inhibition and EC50 are often used to express the quantification of antioxidant properties. The value of the capacity of the antioxidant is expressed by the EC50 value which represents the volume of sample required for 50% reduction of DPPH free radicals [19]. Keran et al. [19] found that the highest value of methanolic extract for 50 mg was 79, 3 %, while with concentration of 40 mg, inhibition value of activity of radicals was not too much lower, 76, 2 %. Other previous conducted studies have stated the alcoholic extracts to possess better antioxidant activity compared to the aqueous ones [17].

In our study, in accordance with the results of the TPC study, the DPPH assay confirmed the higher values in the *Allium ursinum* L. methanolic extract (EC50=66,66-77,21µg/ml) compared to the ethanolic extract (EC50=46,10-77,21 µg/ml), while the lowest values were found in the traditional tincture (EC50=44,99-50,00 µg/ml). We have found a significant correlation between the antioxidant potential and total flavonoid content for the traditional tincture $r=0.99$ $p>0.05$.

Conclusion

Our study concludes that *Allium ursinum* L. (from Montenegro and Bosnia and Herzegovina) leaf extracts have very high antioxidant activity. We have investigated fresh leaves tincture and two different dry leaves extracts. 80% methanol was found to be the most effective extraction solvent for this plant species, while the results for total phenolic, total flavonoid and radical scavenging activity were pretty much the same in the 70% ethanolic extract and the traditional tincture. Also, significant positive correlations was found between the antioxidant activities and the flavonoid content from a traditional tincture ($r=0.99$; $p>0.05$) indicating that these phytochemicals are the major contributors of wild garlic antioxidant capacity. The results of the present study suggest that *Allium ursinum* L. is highly recommended as dietary supplement.

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